Energetics and Application of Heterotrophy in Acetogenic Bacteria

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Acetogenic bacteria are a diverse group of strictly anaerobic bacteria that utilize the Wood-Ljungdahl pathway for CO_2 fixation and energy conservation. These microorganisms play an important part in the global carbon cycle and are a key component of the anaerobic food web. Their most prominent metabolic feature is autotrophic growth with molecular hydrogen and carbon dioxide as the substrates. However, most members also show an outstanding metabolic flexibility for utilizing a vast variety of different substrates. In contrast to autotrophic growth, which is hardly competitive, metabolic flexibility is seen as a key ability of acetogens to compete in ecosystems and might explain the almost-ubiquitous distribution of acetogenic bacteria in anoxic environments. This review covers the latest findings with respect to the heterotrophic metabolism of acetogenic bacteria, including utilization of carbohydrates, lactate, and different alcohols, especially in the model acetogen *Acetobacterium woodii*. Modularity of metabolism, a key concept of pathway design in synthetic biology, together with electron bifurcation, to overcome energetic barriers, appears to be the basis for the amazing substrate spectrum. At the same time, acetogens depend on only a relatively small number of enzymes to expand the substrate spectrum. We will discuss the energetic advantages of coupling CO_2 reduction to fermentations that exploit otherwise-inaccessible substrates and the ecological advantages, as well as the biotechnological applications of the heterotrophic metabolism of acetogens.

Synthesis of acetate from molecular hydrogen (H_2) and carbon dioxide (CO_2) by microorganisms was discovered in 1932 by F. Fischer ([1]). Only 4 years later, the first acetogenic bacterium was isolated in pure culture ([2]). However, understanding the metabolism of acetogenesis turned out to be a challenging task and continues to be so today. Nevertheless, the road toward this goal is paved with landmark discoveries in the field of microbiology. The most characteristic feature of acetogens is their ability to produce acetate from H_2 plus CO_2. This reaction provides only very little energy ($\Delta G^{0'} = -95$ kJ/mol [[3]]); still, acetogens evolved special adaptations to "make a living" from this conversion ([4], [5]). We refer to this as "autotrophic acetogenesis." Ironically, major parts to our understanding of this trait came from studies on an organism that was not known to perform autotrophic acetogenesis: *Moorella thermoacetica* (formerly *Clostridium thermoaceticum*). This organism, isolated by F. E. Fontaine in 1942 ([6]), attracted interest for its ability to convert glucose stoichiometrically to 3 moles of acetate, a conversion named "homoacetogenesis." Elucidation of this pathway led to the discovery of an entirely new pathway of CO_2 fixation, the reductive acetyl coenzyme A (CoA) pathway, or the Wood-Ljungdahl pathway (WLP; named after Harland G. Wood and Lars G. Ljungdahl, who made major contributions to the elucidation of this pathway) ([7]–[9]). This pathway has the unique feature of being the only known pathway of CO_2 fixation that can directly be coupled to net energy conservation ([10]). This has been known for decades; however, only recently was light shed on the question of how this coupling is achieved in the model acetogen, *Acetobacterium woodii*. Contrary to initial expectations, it turned out that none of the enzymes of the WLP is directly involved in energy conservation ([11]–[13]). Instead, the whole pathway is more of an electron sink for redox balancing, instead of a respiratory chain. This has very interesting consequences for the metabolism of acetogens, which we address in this review. The WLP can easily be combined with a vast variety of electron donors, which might be ecologically the most important advantage of acetogens *in situ*. Redox balancing by the WLP in creases the utilizable energy of many substrates and thus provides an energetic advantage over classical fermentations. For some substrates such as ethanol, redox coupling to the WLP is a prerequisite for their utilization in the absence of oxygen or alternative electron acceptors. The outstanding metabolic flexibility is reflected in a very modular organization of the metabolism. Understanding this modularity is also a crucial prerequisite for synthetic biology approaches to utilize the unique abilities of acetogens for biotechnological purposes. Therefore, we will summarize and discuss recent findings on the metabolism, bioenergetics, and energy conservation during utilization of organic substrates, such as carbohydrates, organic acids, and alcohols, by acetogens, with the main focus on *A. woodii*.

WHAT DEFINES AN "ACETOGEN"?

Acetogens are a very diverse group of microorganisms that includes bacteria from many different phyla, most of them able to utilize many different substrates. To define an acetogen, the feature of "acetogenesis" in acetogens must be clearly distinguished from the sole ability to produce acetate as an end product. Many organisms, such as enterobacteria, produce acetate as an end product during carbohydrate fermentation ([14]). Acetic acid bacteria produce acetate as a product from incomplete oxidation of

ethanol (15). In contrast, genuine acetogens are bacteria that can produce acetyl-CoA (and from that, in most cases, acetate as the end product) from two molecules of CO_2 and thus from inorganic carbon, catalyzed by the reactions of the WLP. It is important to note that some acetogens produce no acetate as end product (16); therefore, an unambiguous definition must include acetyl-CoA as a product from two molecules of CO_2 shared by all acetogens, from which the true end products branch off. In addition, for a genuine acetogen, the WLP must be coupled to energy conservation to distinguish it from organisms that utilize the WLP for carbon fixation only. Here, the term acetogenesis is used in reference to organisms that fulfill all the above-mentioned requirements. For a further discussion of this topic, we refer readers to an excellent chapter on past and current perspectives by H. Drake in the book *Acetogenesis* (17).

Acetogenesis is not a phylogenetic trait and can be found in many different phyla, with several acetogens clustered in the class *Clostridia* within the phylum *Firmicutes* (18, 19). Many of the closest relatives of acetogenic species do not share the capability of acetogenesis, implying that this feature can either be easily acquired by horizontal gene transfer or was lost many times independently during evolution. The second scenario is supported by the current theory of acetogens as the autotrophic origin of bacteria (20). Coupling acetogenesis to the conversion of many substrates available in anoxic ecosystems results in improved energy gain in comparison to classic fermentations and can still provide an evolutionary advantage in ecosystems scarce in external electron acceptors, even if autotrophic carbon fixation is not necessary.

The phylogeny of acetogens does not imply a special classification in different subgroups. However, acetogens can be classified into two subgroups based on the distinct enzymes responsible for energy conservation (5). The best-understood organism in terms of energy conservation is the Gram-positive acetogen *A. woodii*, which belongs to the order *Clostridiales* (21). It utilizes a membrane-bound, sodium ion-translocating ferredoxin:NAD$^+$ oxidoreductase, most likely encoded by *rnf* genes, for the buildup of a sodium ion gradient that is subsequently utilized by a sodium ion-dependent ATP synthase for the synthesis of ATP (22–25). As a result, the presence of genes coding for an Rnf complex in the genome of an acetogen has been used to classify an organism as "Rnf-dependent" acetogen (4). All acetogens that lack such genes have been found to encode a membrane-bound, ion-translocating, energy-converting hydrogenase (Ech) (12, 26, 27). The details of energy conservation in these organisms are still uncertain; however, the present evidence supports the notion to classify them as "Ech-dependent" acetogens. Due to the lack of experimental data for the latter organisms, in the following discussion on energy conservation during heterotrophic growth we will only address Rnf-dependent acetogens and especially *A. woodii*.

WOOD-LJUNGDAHL PATHWAY AND REDOX BALANCING

Constitutive parts of the WLP have been elucidated in the Ech-containing acetogen *M. thermoacetica* (12, 28). Now, available data indicate that the basic principles can be transferred to all acetogens, including those in the Rnf-containing subclass. The WLP is a linear pathway with two converging branches that catalyze the reduction and merging of 2 molecules of CO_2, resulting in the formation of 1 molecule of acetyl-CoA (Fig. 1). Important for further discussion here is the strict definition of the WLP as a
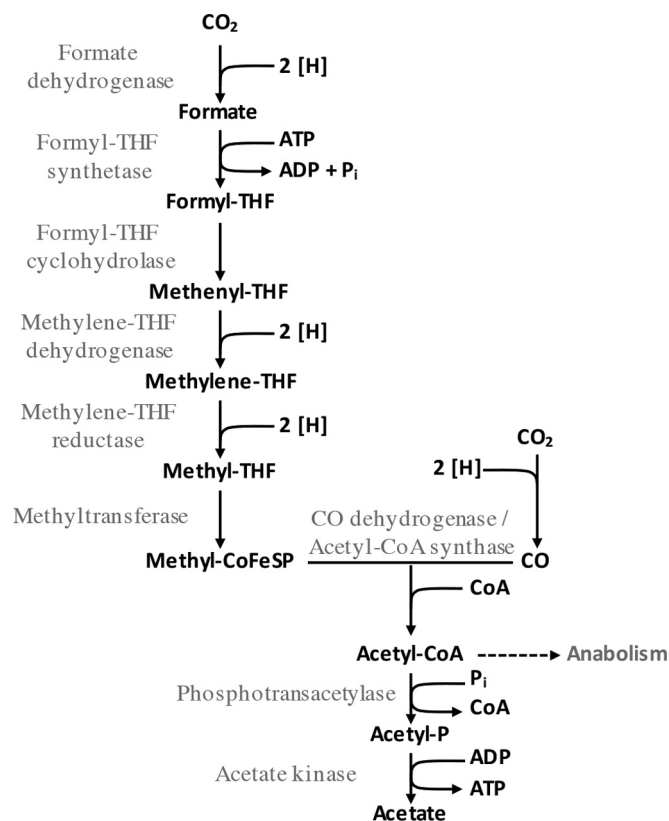

FIG 1 The Wood-Ljungdahl pathway of acetogenic bacteria. [H], redox equivalent (one electron + one proton); THF, tetrahydrofolate; CoFe-SP, corrinoid iron-sulfur protein. In *A. woodii*, the utilized redox equivalents are H_2 for the formate dehydrogenase step, NADH for the methylene-THF dehydrogenase and methylene-THF reductase steps, and reduced ferredoxin for the CO dehydrogenase/acetyl-CoA synthase step.

module, including only those reactions that lead from the 2 molecules of CO_2 to acetyl-CoA. Other reactions, such as H_2 oxidation by a hydrogenase, are not essential parts of the WLP and can be replaced by other electron-providing reactions; they are thus not included in this module. The WLP can be further subdivided into two converging branches: in the methyl branch, CO_2 is reduced to formate, which is subsequently bound to the cofactor tetrahydrofolate and reduced stepwise to a tetrahydrofolate-bound methyl group, which serves as the precursor of the methyl group of acetyl-CoA. In the carbonyl branch, a second molecule of CO_2 is reduced to enzyme-bound carbon monoxide (CO), serving as the precursor for the carbonyl group of acetyl-CoA, a reaction catalyzed by the key enzyme of the WLP, the carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/Acs) (8). This enzyme also catalyzes the synthesis of acetyl-CoA from the methyl group, CO, and coenzyme A by an enzymatic mechanism that involves several organometallic intermediates (29). In advance of this reaction, the methyl group is transferred from the cofactor tetrahydrofolate to CODH/Acs by a corrinoid-containing methyltransferase (30, 31). The product acetyl-CoA can then be incorporated into biomass or converted to end products, typically acetate (via phosphotransacetylase and acetate kinase [32, 33]).

In all acetogens examined to date, all enzymes of the WLP are soluble cytoplasmic enzymes, and thus no enzyme is directly involved in chemiosmotic energy conservation. Moreover, the WLP

includes one step of ATP synthesis via substrate-level phosphorylation (SLP), namely, the synthesis of acetate from acetyl phosphate (33), which is balanced by one ATP-consuming step, namely, the fusion of formate to the cofactor tetrahydrofolate (34), which results in no net ATP gain of the complete pathway by SLP. What is the function of the WLP then? During autotrophic growth, one function of the WLP is CO_2 fixation for the formation of biomass. However, during heterotrophic growth, this function is in most cases dispensable, and since we are dealing with heterotrophy in this review, we want to neglect the autotrophic function of the WLP. In addition to CO_2 fixation, the WLP has an important function during energy conservation: every chemotrophic organism conserves energy from the oxidation of an energy-rich substrate, such as glucose, and must reoxidize the resulting reduced electron carriers, such as NADH, to obtain a balanced redox stoichiometry. This can either be achieved by transferring the electrons to an external electron acceptor, such as oxygen, or in the case of fermentations, transferring them to an incompletely oxidized intermediate, such as pyruvate or acetyl-CoA. The latter provides the least amount of energy for the organisms from a given substrate (35). In the WLP, the reduction of 2 molecules of CO_2 to acetic acid requires 8 reducing equivalents (1 reducing equivalent, i.e., [H], equals 1 electron plus 1 proton), and this pathway therefore performs the function of reoxidizing reduced electron carriers. The reducing equivalents reoxidized in *A. woodii* by the WLP for the reduction of 2 molecules of CO_2 consist of 2 NADH, 1 reduced ferredoxin, and 1 H_2 (Fig. 1). Reoxidizing the electron carriers by the WLP allows further oxidation of the substrate (for example, to acetate via acetate kinase) and therefore the release of more energy that can be utilized by the cell. For some substrates, this requires the external supply of CO_2 as the substrate for the WLP; however, this molecule is distributed ubiquitously in anoxic ecosystems. Furthermore, the oxidation of many substrates utilized by acetogens releases CO_2, so the electron acceptor is provided during oxidation of the substrate. This is comparable to the utilization of protons as electron acceptor by some fermenting organisms, such as *Thermotoga maritima* or *Clostridium pasteurianum* (36, 37). Protons are ubiquitously available, and proton reduction increases ATP gain. However, the redox potential of the redox couple H^+/H_2 ($E^{0'} = -414$ mV) is, even at physiological H_2 partial pressures, more negative than that of CO_2/acetate ($E^{0'} = -290$ mV), resulting in energetic restriction of the reactions that can be coupled to proton reduction, in contrast to CO_2 reduction (3).

COUPLING ENERGY CONSERVATION TO THE WLP

It has been assumed for a long time that enzymes of the WLP are directly involved in energy conservation. However, it turned out that this is not the case. Instead, a ferredoxin:NAD^+ oxidoreductase activity has been discovered as the only coupling site of energy conservation in the model acetogen *A. woodii* (38, 39). This activity is most likely catalyzed by a membrane-bound multisubunit Rnf complex that couples the electron transfer from reduced ferredoxin to NAD^+ to the translocation of sodium ions across the cytoplasmic membrane (11, 39). It has been shown for *A. woodii* that electron transfer from reduced ferredoxin to NAD^+ is coupled to the generation of a primary electrogenic sodium ion potential (24). Interestingly and of important physiological implications, the Rnf complex can also drive endergonic reverse electron transfer from NADH to oxidized ferredoxin, at the expense of the

electrochemical sodium ion potential (25). As described later, this reaction is essential for the conversion of important organic substrates. The discovery of the Rnf complex as a coupling site shifted the site of energy conservation one step back in the hierarchy of electron transfers: ATP synthesis is not coupled to the electron transfer from a soluble electron carrier such as NADH to a terminal electron acceptor (O_2, nitrate, fumarate) but to the electron transfer between two soluble electron carriers. Transfer of electrons from reduced ferredoxin to NAD^+ is coupled to chemiosmotic ion translocation and thus ATP synthesis. This process does not involve the electron transfer to the final electron acceptor CO_2. This view can be further expanded to respiratory chains in general: in most organisms chemiosmosis is driven by the electron transfer from NADH to a final acceptor, such as oxygen, nitrate, or fumarate. In the hierarchy of redox potentials, acetogens utilize one step before, expanding the redox range of known respiratory chains to a more negative range. The end product of chemiosmotic energy conservation in acetogens is NADH. Due to a lack of an appropriate electron acceptor, NADH is not used for further energy conservation but simply reoxidized in an ATP-neutral process by the WLP.

In summary, we can separate the catabolism of *A. woodii* into three independent modules (Fig. 2). The WLP can be regarded as an energy-neutral, cytoplasmic enzyme system for reoxidation of NADH and reduced ferredoxin (Fig. 2C). This module can be combined for the oxidation of a large variety of organic and inorganic substrates as described below (Fig. 2A). Electron-accepting and electron-generating modules are connected by the Rnf complex (Fig. 2B). This enzyme balances the redox pools of NAD^+/NADH and ferredoxin (ox/red). The redox potential of NAD^+/NADH is -320 mV under standard conditions and more in the range of -280 mV under physiological conditions (3, 40). The redox potential of ferredoxin (ox/red) is between -400 and -500 mV under physiological conditions (10, 41, 42). Therefore, electron transfer from reduced ferredoxin to NAD^+ can drive the generation of a sodium ion gradient and thus provides additional ATP. Accordingly, the reverse reaction consumes ATP.

METABOLIC FLEXIBILITY OF ACETOGENS

All acetogens have in common that they contain the WLP for the reduction of 2 molecules CO_2 to acetyl-CoA. Beyond that, acetogens show a remarkable metabolic diversity. Most of them can use H_2 as electron donor for CO_2 reduction and carbon fixation and thus can live autotrophically. However, in some cases, this ability is easily overlooked, because autotrophic growth is often very poor. For *M. thermoacetica*, autotrophic growth with H_2 and CO_2 was discovered more than 40 years after its first description (43). As will be described later, autotrophic growth is a very uncompetitive feature, e.g., in competition with methanogens, and does not explain the ubiquitous distribution of acetogens. Instead, the heterotrophic potential makes acetogens the most metabolically diverse group of anaerobes (44). Besides H_2, they can utilize a vast number of electron donors. These donors include hexoses like glucose or fructose, which most acetogens can use, but also pentoses like xylose (44). Alcohols, including ethanol, propanol, and butanol, can be utilized, for example, by *M. thermoacetica*, whereas diols such as 1,2-propanediol, ethylene glycol, or 2,3-butanediol can be utilized, for example, by some *Acetobacterium* species, including *A. woodii* (28, 45, 46). The potential substrates
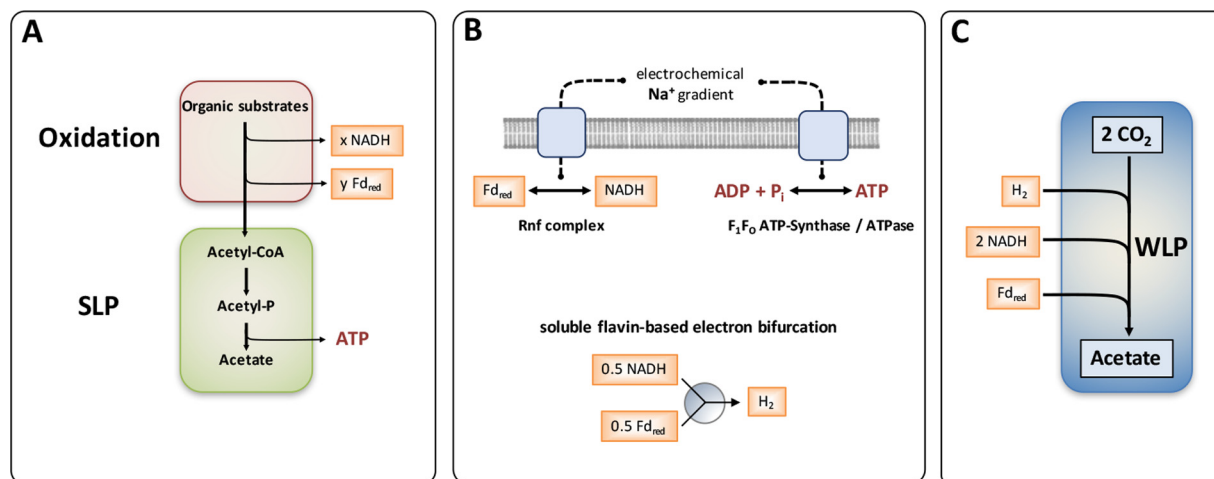
FIG 2 The heterotrophic metabolism of *A. woodii*, the model organism of the Rnf-dependent acetogens, is composed of well-defined modules. (A) Most heterotrophic substrates (e.g., glucose, lactate, ethanol) are oxidized to the level of acetyl-CoA by transferring the electrons to NAD^+ and ferredoxin. Acetyl-CoA is converted to acetate, generating 1 ATP by SLP. (B) The module responsible for redox balancing between modules A and C. This is achieved either by a soluble hydrogenase using flavin-based electron bifurcation or by a membrane-bound Rnf complex. The latter is coupled to ATP synthesis via an electrochemical sodium ion gradient and a Na^+-dependent F_1F_O ATP synthase/ATPase. Note that some acetogens have Ech instead of Rnf, and in both, Ech- and Rnf-containing species, the coupling ion may be Na^+ or H^+. (C) The WLP functions as an electron sink to reoxidize the electron carriers by reducing CO_2 to acetate. Fd_{red}, reduced ferredoxin (reduced by 2 electrons).

of acetogens extend to carboxylic acids, such as pyruvate, lactate, citrate, or the C_2 acids oxalate, glyoxylate, or glycolate. In addition, the WLP is perfectly suited to metabolize C_1 compounds, such as methanol, formate, or CO (44). Some acetogens, such as different *Sporomusa* species, seem to be specialized for the utilization of C-1 groups from methylated compounds, such as betaine, sarcosine or *N*,*N*-dimethylglycine, or can even utilize abiotic electron sources such as Fe(0) (47, 48).

Considering the electron acceptors, CO_2 is not an essential acceptor, and many acetogens can utilize alternative electron acceptors such as nitrate, fumarate, phenylacrylates, or perchlorate (28, 49, 50). Most alternative electron acceptors provide more energy than CO_2 and when provided simultaneously are preferred over acetogenesis. Therefore, many acetogens can be viewed as facultative acetogens that use acetogenesis in the absence of better electron acceptors (9). The flexibility gets even more obvious when mixotrophic growth is taken into consideration. It has been shown for some acetogens that they can utilize different substrates simultaneously, for example, H_2 and CO_2 together with glucose, or H_2 and CO_2 together with phenylacrylates (51, 52). In addition, interspecies H_2 transfer can be used instead of acetogenesis to get rid of surplus reducing equivalents, as shown for *A. woodii* growing together with an H_2-consuming methanogen. In this scenario, *A. woodii* produces only 2 mol acetate per mol glucose, and the reducing equivalents are used for methanogenesis by the partner organism (53).

A detailed description of all these metabolic capacities would reach far beyond the scope of this review. However, most heterotrophic substrates follow a general scheme and share common features. In the following sections, we discuss the metabolism of four exemplary heterotrophic substrates that have been elucidated in the model acetogen, *A. woodii*, in detail: carbohydrates, lactate, ethanol, and 2,3-butanediol. For a further discussion of the autotrophic capacities, we refer readers to our recent review on this topic (4).

CARBOHYDRATE FERMENTATION

In the absence of external electron acceptors, carbohydrates such as glucose can provide energy if converted to incompletely oxidized end products, such as ethanol, lactate, or acetate, by fermentation. Most of the energy stored in glucose remains in the end product; thus, only a fraction is available for ATP generation. For example, glucose conversion to ethanol has the stoichiometry shown in reaction 1.

$$\text{glucose} \rightarrow 2 \text{ ethanol} + 2\ CO_2 \quad (1)$$
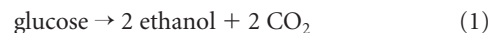
This reaction releases −230 kJ/mol glucose (54, 55). During this conversion, 2 mol ATP/mol glucose is generated during the reactions of the Embden-Meyerhof-Parnas pathway (glycolysis). Around 3.3 mol ATP/mol glucose is generated during butyric acid fermentation as carried out by strictly anaerobic clostridia, such as *Clostridium pasteurianum*, forming the end products acetate, butyrate, CO_2, and H_2 (42). Up to 4 mol ATP/mol glucose can be generated when glucose is completely converted to acetate, CO_2, and H_2, for example, via catalysis by the rumen bacterium *Ruminococcus albus* (56). However, this high ATP gain is only achieved when the organism is grown together with a hydrogen-consuming partner. In contrast, acetogens do not usually produce H_2 in stoichiometric amounts as an end product, but instead convert glucose completely to 3 mol of acetate according to reaction 2 ($\Delta G^{0\prime}$ of −311 kJ/mol glucose).

$$\text{glucose} \rightarrow 3 \text{ acetate} \quad (2)$$

This process is called homoacetate fermentation ("homoacetogenesis") and provides the highest known ATP gain for glucose fermentations, more than 4.3 ATP/mol glucose in the case of *A. woodii* (35). This bacterium uses the Embden-Meyerhof-Parnas pathway for the oxidation of 1 mol glucose to 2 mol pyruvate (Fig. 3A) (11). Thereby, 2 ATP are synthesized by SLP. Pyruvate is oxidatively decarboxylated by the enzyme pyruvate:ferredoxin oxidoreductase (PFO) to acetyl-CoA and CO_2 (57). Both acetyl-
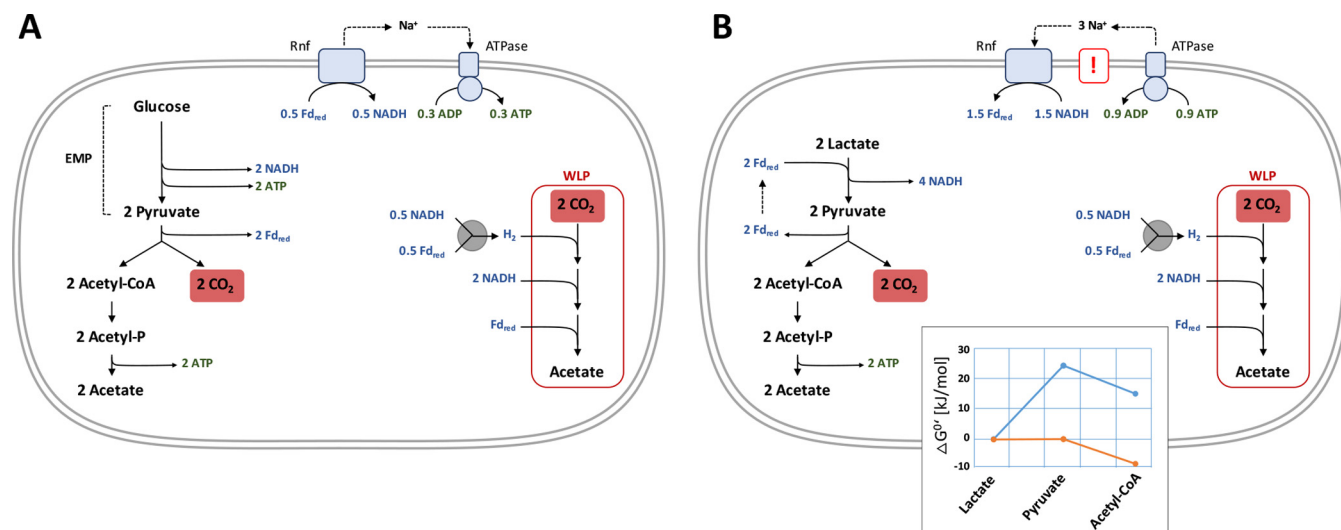
FIG 3 Carbohydrate (A) and lactate (B) metabolism of *A. woodii*. The inset in panel B represents the energetics ($\Delta G^{0'}$ profile for the conversion of the corresponding substrates to the products/intermediates) of the oxidation of lactate to acetyl-CoA not coupled (blue) or coupled (orange) to flavin-based electron bifurcation. Blue line, lactate is oxidized with NAD^+ as the electron acceptor, followed by ferredoxin-dependent oxidation of pyruvate. Orange line, lactate is oxidized with NAD^+ as the electron acceptor, coupled to NAD^+ reduction by reduced ferredoxin by electron bifurcation, and pyruvate is oxidized with ferredoxin as the electron acceptor. EMP, Embden-Meyerhof-Parnas pathway (glycolysis); Fd_{red}, reduced ferredoxin (reduced by 2 electrons).

CoA molecules are converted to acetate, yielding 2 additional ATP by SLP and resulting in a conversion of glucose according to reaction 3 (where [H] represents 1 electron plus 1 proton).

$$\text{glucose} + 4\ ADP + 4\ P_i \rightarrow$$
$$2\ \text{acetate} + 2\ CO_2 + 4\ ATP + 2\ H_2O + 8\ [H] \quad (3)$$
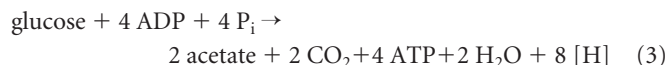
This high ATP yield is only possible because the redox balance is maintained by subsequent reduction of both molecules of released CO_2 to a third molecule of acetate via the WLP, according to reaction 4.

$$2\ CO_2 + 8\ [H] \rightarrow \text{acetate} + 2\ H_2O \quad (4)$$

In *A. woodii*, conversion of glucose to 2 acetates results in generation of 2 NADH and 2 reduced ferredoxins (each ferredoxin is assumed to accept 2 electrons) (Fig. 3A). The WLP utilizes 1 ferredoxin, 2 NADH, and 1 H_2 (or a second ferredoxin) (4). Therefore, one H_2 must be produced from 1 reduced ferredoxin to balance the oxidative module (glycolysis plus PFO) and the reductive module (WLP). Two additional enzymes are necessary to accomplish this task: the Rnf complex and an electron-bifurcating hydrogenase (58). The term flavin-based electron bifurcation describes a novel mode of energetic coupling of two redox reactions catalyzed by soluble enzyme complexes. An endergonic electron transfer reaction is achieved by coupling it to an exergonic electron transfer from the same electron donor to a second acceptor (42, 59). As we discuss below, this concept is an essential feature of the metabolism of acetogens and allows conversions that are otherwise not possible. Coming back to carbohydrate metabolism of *A. woodii*, the Rnf complex transfers the electrons from 0.5 ferredoxin to 0.5 NAD^+. Afterwards, the electron-bifurcating hydrogenase can produce molecular hydrogen according to reaction 5,

$$0.5\ NADH + 0.5\ \text{ferredoxin(red)} + 0.5\ H^+ \leftrightarrows$$
$$H_2 + 0.5\ NAD^+ + 0.5\ \text{ferredoxin(ox)} \quad (5)$$
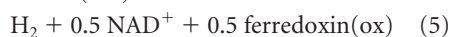
and thus balances all redox reactions. In the course of the electron transfer from reduced ferredoxin to NAD^+, the Rnf complex translocates 1 sodium ion across the cytoplasmic membrane (1 Na^+/electron). The amount of ATP that can be synthesized from this electrochemical sodium ion gradient can be calculated from the ion-to-ATP stoichiometry of the ATP synthase. In *A. woodii*, the membrane-embedded rotor subunit (called the *c*-ring) has 10 ion binding sites (60, 61). One complete rotation of this subunit is coupled to the synthesis of 3 ATP, resulting in an ion-to-ATP stoichiometry of 3.3. Therefore, 1 sodium ion translocated by the Rnf complex is sufficient for the synthesis of 0.3 ATP. In sum, homoacetate fermentation in *A. woodii* results in the formation of 4 ATP by SLP and 0.3 additional ATP by chemiosmotic energy conservation via the Rnf complex and the membrane-bound ATP synthase, and therefore the highest reported ATP yield for carbohydrate fermentation of 4.3 mol ATP per mol of glucose.

It is interesting to consider not only the ATP yield of a metabolic pathway (in *n* moles of ATP formed per *n* moles of substrate consumed) but also the thermodynamic efficiency. This value describes how much of the energy that is released from a given reaction is conserved in the form of ATP. A higher thermodynamic efficiency might provide an ecological advantage against rivals competing for the same substrate. Most anaerobic metabolisms work at thermodynamic efficiencies of 20 to 50% (3). Homoacetogenesis from glucose as carried out by *A. woodii* has an efficiency of 61% (calculated from the number of ATP molecules synthesized times the 43.9 kJ/mol necessary for ATP synthesis under physiological concentrations [3] [we use this value for the calculations for comparability despite the fact that the phosphorylation potential in *A. woodii* has been determined experimentally to be 30 to 38 kJ/mol, depending on the substrate {62}] divided by the released amount of energy of the whole reaction). So, it has not only a very high ATP yield of 4.3 mol ATP/mol glucose but also a very high thermodynamic efficiency compared, for example, to ethanol formation from glucose, as described above, with an efficiency of 38% or homolactic fermentation of 47% (calculated from the $\Delta G^{0'}$ of -188.6 kJ/mol for the conversion of 1 glucose to

2 lactate ([54], [55]) divided by the $\Delta G^{0'}$ value for the synthesis of 2 ATP in this fermentation [35]). We discuss below that this is the case for most metabolic pathways of *A. woodii* and is a result of flavin-based electron bifurcation as a novel mechanism of coupling exergonic and endergonic redox reactions that increases the thermodynamic efficiency, as has also been described for nonacetogenic bacteria ([42]).

Homoacetate fermentation clearly illustrates the modularity of the metabolism of acetogens and the resulting benefits. The WLP is added as an isolated module to the classic reactions of glucose fermentation via glycolysis. This allows the complete oxidation of 1 glucose to 3 acetate. Otherwise, the redox balance must be maintained, for example, by reduction of pyruvate to ethanol or lactate, which is not coupled to ATP synthesis via SLP. The unbalanced pools of the electron carriers are adjusted by the Rnf complex. Coupling this reaction to the synthesis of even more ATP is just the icing on the cake for this very energy-efficient fermentation. In the natural setting, the high ATP yield should allow acetogens to outcompete other microorganisms if carbohydrates, but no suitable electron acceptor, are present. In ecosystems, comparable ATP yields can otherwise only be obtained by syntrophic associations of fermenting organisms together with an H_2-consuming methanogen ([63], [64]). In this situation, 1 glucose is converted to 3 acetate, 2 CO_2, and 4 H_2 by the fermenter. The methanogen subsequently utilizes 4 H_2 and 1 CO_2 to produce 1 methane, thus keeping the H_2 pressure low enough to facilitate the reaction of the fermenting organism.

The described general scheme of the metabolism of acetogens is preserved for a large variety of organic substrates and increases the ATP yield beyond that of fermentations without the WLP. Different substrates are oxidized to the level of acetyl-CoA, followed by conversion to acetate coupled to ATP synthesis by the acetate kinase ([Fig. 2]). The electron carriers are reoxidized by the WLP. Connection of the oxidative module and the WLP is facilitated by the Rnf complex. This reaction is not in all cases coupled to additional energy conservation, but it can also be used to provide the energy for endergonic "reverse" electron transfer reactions, as we discuss in the next section for the unfavorable oxidation of lactate.

LACTATE METABOLISM

Many acetogens can utilize lactate as a carbon and energy source and convert it exclusively to acetate according to reaction 6 ($\Delta G^{0'}$ of −61 kJ/mol lactate [[54], [55]]).

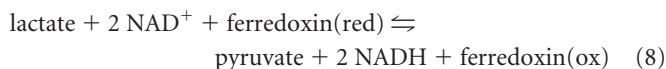$$2 \text{ lactate} \rightarrow 3 \text{ acetate} \qquad (6)$$

This fermentation has been known for decades; however, it was only recently that the corresponding pathway was understood in detail ([65]). Lactate metabolism is a genuine fermentation, as is homoacetogenic fermentation of glucose; even so, CO_2 is reduced as the electron acceptor. In general, 2 lactate are oxidized to 2 pyruvate by a lactate dehydrogenase (LDH), followed by an oxidative decarboxylation to 2 acetyl-CoA and 2 CO_2 ([Fig. 3B]) ([9], [19]). As described before, both acetyl-CoA molecules are converted to acetyl-phosphate by a phosphotransacetylase, followed by a transfer of the phosphate to ADP by an acetate kinase, resulting in the formation of 2 acetate and 2 ATP molecules. To reoxidize the electron carriers that have been reduced in this process, both generated CO_2 molecules are reduced to acetate via the WLP. This scheme illustrates that there is no need for an external supply

of CO_2 as an external electron acceptor, a prerequisite for a fermentation in the strict sense. For *A. woodii*, this has also been shown experimentally (V. Müller, unpublished data). At first appearance, lactate fermentation by acetogens is a simple metabolism, following the scheme presented above: oxidation of an organic substrate and coupling of this reaction to the WLP via an electron-accepting module. However, the pathway contains unfavorable thermodynamic barriers. The redox potential of lactate/pyruvate is very positive ($E^{0'} = -190$ mV [3]). Therefore, electron transfer to NAD^+ ($E^{0'} = -320$ mV) is a highly endergonic reaction (reaction 7; $\Delta G^{0'}$ of +25 kJ/mol).

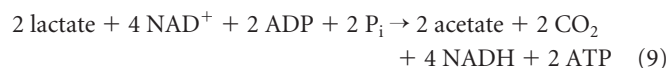$$\text{lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH \qquad (7)$$

The next reaction, oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2, is coupled to ferredoxin reduction in an only slightly exergonic reaction (−9.6 kJ/mol) and is thus not able to shift the equilibrium of the first one ([Fig. 3B], inset). Some aerobic bacteria circumvent the problem by using a membrane-bound LDH with ubiquinone as the electron acceptor for the first reaction ([66]–[68]). If lactate is oxidized with an NAD^+-dependent LDH, for example in human cells, replacement of ferredoxin as electron acceptor of oxidative decarboxylation with NAD^+, a reaction catalyzed by the pyruvate dehydrogenase complex, can shift the equilibrium. This reaction is highly exergonic ($\Delta G^{0'} = -40$ kJ/mol [[54], [55]]), and thus the the concentration of the intermediate pyruvate is decreased enough to force lactate oxidation. *A. woodii* uses a completely different approach to facilitate the endergonic oxidation of lactate with NAD^+ as electron acceptor: flavin-based electron bifurcation ([65]). The endergonic oxidation of lactate with NAD^+ as electron acceptor is coupled to the exergonic oxidation of reduced ferredoxin with NAD^+ as electron acceptor. Therefore, one favorable redox reaction is used to drive another unfavorable redox reaction. Upon discovery of this mechanism, flavin-based electron bifurcation was defined as oxidation of one substrate that leads to the energetically coupled reduction of two different electron acceptors by a single enzyme ([42], [59], [69]). The path of the electrons is therefore bifurcated or forked, leading to the term bifurcation. In the case of lactate oxidation, the bifurcation reaction is catalyzed in the reverse direction. To emphasize this direction, the term "electron confurcation" was coined ([70]). However, electron bifurcation and confurcation describe the same enzyme mechanism; the two terms just considering the two different directions of the catalyzed reaction.

The LDH of *A. woodii* has been purified and characterized ([65]). It is an enzyme complex of an LDH and two electron-transferring flavoproteins (EtfAB). The enzyme complex coordinates 3 flavins and 1 iron sulfur cluster. In analogy to other electron-bifurcating enzyme complexes, namely, butyryl-CoA dehydrogenase/EtfAB and caffeyl-CoA reductase/EftAB ([69], [71]), the bifurcation reaction was assigned to the subunits EtfAB. Oxidation of 1 lactate releases 2 electrons that could be transferred from the LDH subunit to the subunits of EtfAB. Two additional electrons are derived from oxidation of 1 ferredoxin. All 4 electrons could then be used to reduce 2 NAD^+. Coupling of exergonic reduction of NAD^+ by reduced ferredoxin to the endergonic reduction of NAD^+ by lactate renders the overall reaction energy neutral (reaction 8; $\Delta G^{0'}$ of 0 kJ/mol).

$$\text{lactate} + 2 NAD^+ + \text{ferredoxin(red)} \rightleftharpoons$$
$$\text{pyruvate} + 2 NADH + \text{ferredoxin(ox)} \qquad (8)$$

The elucidation of the reaction mechanism of lactate oxidation in *A. woodii* allows a stoichiometric reflection of the complete oxidative module of acetogenic lactate fermentation. Two lactate are oxidized to 2 pyruvate in a reaction utilizing 2 reduced ferredoxin and generating 4 NADH. Pyruvate is subsequently oxidatively decarboxylated to acetyl-CoA and CO_2 coupled to ferredoxin reduction, thus reducing both ferredoxins needed for the first reaction. Afterwards, 2 acetyl-CoA are converted to acetate, coupled to synthesis of 2 ATP. In sum, lactate is oxidized according to reaction 9.

$$2 \text{ lactate} + 4 \text{ NAD}^+ + 2 \text{ ADP} + 2 \text{ P}_i \rightarrow 2 \text{ acetate} + 2 \text{ CO}_2 + 4 \text{ NADH} + 2 \text{ ATP} \quad (9)$$

In accordance with the general scheme introduced for the heterotrophic metabolism of *A. woodii*, NADH is reoxidized by reduction of both CO_2 molecules to acetate by the WLP. The WLP serves here again just as an isolated module for redox balancing. Again, in *A. woodii* the WLP utilizes 2 NADH, 1 reduced ferredoxin, and 1 H_2 to reduce 2 CO_2. Therefore, this time, 1 reduced ferredoxin and 1 H_2 must be generated from 2 NADH to balance the electron flow. In contrast to homoacetate fermentation of glucose, this time electron flow from the oxidative module to the reductive module is an endergonic reaction and necessitates the input of energy. The electron-bifurcating hydrogenase can produce the necessary H_2 from 0.5 reduced ferredoxin and 0.5 NADH. In sum, 1.5 ferredoxin must be reduced from 1.5 NADH. This reaction is catalyzed by the Rnf complex. So far, the Rnf complex was described as an enzyme used for energy conservation as it couples the exergonic electron flow from reduced ferredoxin to NAD$^+$ to the generation of a transmembrane sodium ion gradient. This time, the Rnf complex has to operate in reverse. The endergonic electron transfer from NADH to ferredoxin is driven by the consumption of a sodium ion gradient established by ATP hydrolysis. Thus, the entire process can be regarded as reverse chemiosmosis: ATP hydrolysis drives the generation of an electrochemical transmembrane ion gradient that then drives reverse electron flow to reduce ferredoxin at the expense of NADH. The Rnf complex can indeed catalyze this reaction, as has been shown experimentally (25). At inverted membrane vesicles, a sodium ion gradient established by ATP hydrolysis was sufficient to energize the electron electron transfer from NADH to ferredoxin.

The complete pathway of lactate fermentation in *A. woodii* is shown in Fig. 3B. Lactate is converted to acetate according to reaction 10.

$$2 \text{ lactate} + 1.1 \text{ ADP} + 1.1 \text{ P}_i \rightarrow 3 \text{ acetate} + 1.1 \text{ ATP} + 1.1 \text{ H}_2\text{O} \quad (10)$$
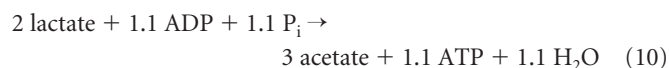
In sum, 0.55 mol ATP is synthesized per lactate. Regarding a released energy of −61 kJ/mol lactate, the thermodynamic efficiency of 39% (calculated from the number of ATP molecules synthesized multiplied by the 43.9 kJ/mol necessary for ATP synthesis under physiological concentrations [3] divided by the released amount of energy) is low in this pathway compared to an efficiency of 61% for homoacetogenesis from glucose. This might be a result of the very steep energy barriers that this pathway has to overcome. Nevertheless, lactate fermentation in *A. woodii* is another fascinating example of the importance of flavin-based electron bifurcation for energy-deprived metabolic pathways. Driving the unfavorable oxidation of lactate to pyruvate by ATP hydrolysis would consume more ATP than produced in the complete path-

way. Therefore, in this case, electron bifurcation is not primarily responsible for increasing the ATP yield or the thermodynamic efficiency of an otherwise-less-efficient conversion, but it is a prerequisite to allow lactate oxidation by *A. woodii*.
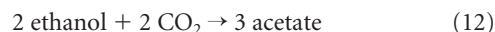
ALCOHOL METABOLISM

Glucose and lactate are substrates that can be fermented via different pathways by many organisms. Coupling the fermentation to the WLP just increases the ATP gain per substrate amount consumed, as we have seen with the two pathways already described. Other substrates, such as ethanol, cannot be fermented by a single organism in the absence of suitable external electron acceptors. Coupling oxidation of these substrates to CO_2 reduction thus allows growth with otherwise-not-exploitable substrates. In this case, CO_2 must be provided externally; however, it is an almost ubiquitously available substrate in anoxic ecosystems.

In general, ethanol oxidation to acetate and H_2 is an endergonic reaction and does not support growth of a single organism. Oxidation of ethanol leads to acetaldehyde and subsequently acetyl-CoA, which can be used to generate 1 ATP via the conversion to acetyl-phosphate and finally acetate. The redox potential of acetate/ethanol is roughly −400 mV (54, 55). Fermentation requires balancing of this half-reaction (reaction 11; $\Delta G^{0'}$ of +9 kJ/mol), which can be achieved in this case only by proton reduction. However, the overall reaction is unfavorable under standard conditions.

$$\text{ethanol} + \text{H}_2\text{O} \rightleftharpoons \text{acetate} + 2 \text{ H}_2 \quad (11)$$

This thermodynamic barrier can only be overcome by syntrophic association of an ethanol-fermenting organism with an H_2-consuming partner organism. The second organism lowers the H_2 concentration, thus shifting the redox potential of H_2 to more-positive values and thus allowing ethanol oxidation by the first organism (63, 72). The classical example for such cooperation is the association of the H_2-consuming methanogen *Methanobacterium bryantii* and the ethanol-oxidizing bacterium called the "S organism" (formerly known as *Methanobacillus omelianskii*) (73, 74).

Acetogens overcome the thermodynamic restriction of ethanol oxidation by replacing protons as the electron acceptor with CO_2. Following the general scheme of the metabolism of acetogens, ethanol metabolism can be clearly separated into an oxidative module and the WLP as the reductive module, coupled via the Rnf complex (Fig. 4A). Acetogens typically convert ethanol and CO_2 to acetate according to reaction 12 ($\Delta G^{0'}$ of −40 kJ/mol ethanol [54, 55]).

$$2 \text{ ethanol} + 2 \text{ CO}_2 \rightarrow 3 \text{ acetate} \quad (12)$$

Just recently, ethanol metabolism was elucidated in detail for the first time for an acetogenic bacterium (75). The oxidative module has a thermodynamic barrier comparable to lactate oxidation. Ethanol oxidation coupled to NAD$^+$ reduction is a highly unfavorable reaction (reaction 13; $\Delta G^{0'}$ of +22 kJ/mol ethanol).

$$\text{ethanol} + \text{NAD}^+ \rightleftharpoons \text{acetaldehyde} + \text{NADH} \quad (13)$$

Analysis of this reaction in *A. woodii* revealed that the energetic problem is solved in a completely different way in comparison to lactate oxidation, even though the redox potential of acetaldehyde/ethanol of −197 mV is almost the same as that of the pyruvate/lactate pair of −190 mV. Whereas flavin-based electron bi-
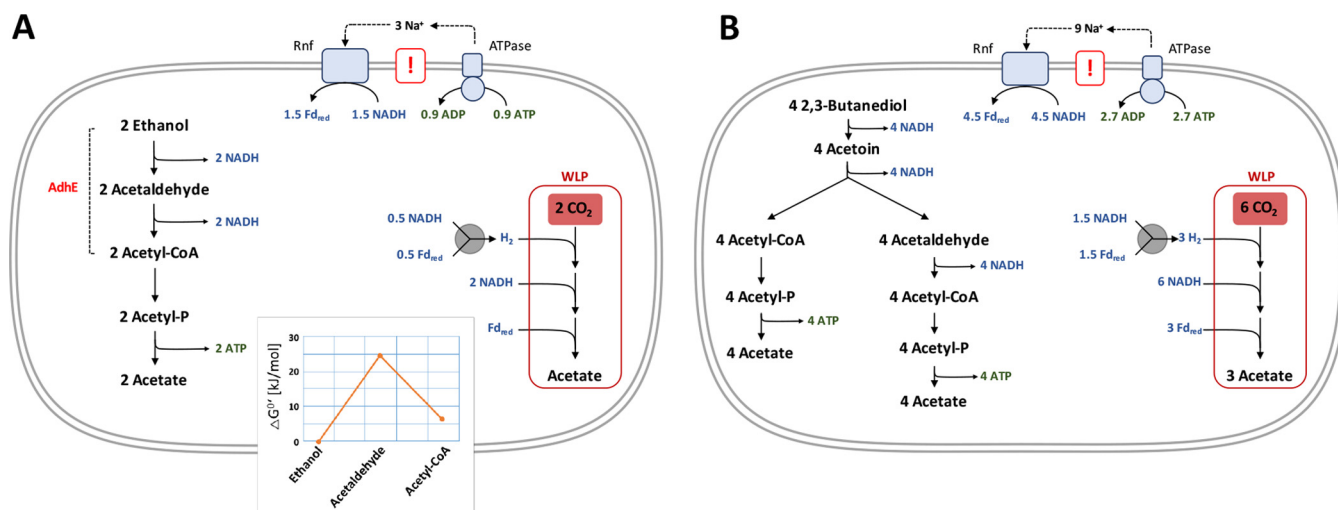
FIG 4 Ethanol (A) and 2,3-butanediol (B) metabolism of *A. woodii*. The inset in panel A represents the energetics ($\Delta G^{0\prime}$ profile for the conversion of the corresponding substrates to the products/intermediates) of the oxidation of ethanol to acetyl-CoA. Both ethanol and acetaldehyde are oxidized with NAD^+ as the electron acceptor. AdhE, bifunctional acetaldehyde/ethanol dehydrogenase; Fd_{red}, reduced ferredoxin (reduced by 2 electrons).

furcation is used to energize the unfavorable oxidation of lactate, ethanol oxidation does not utilize electron bifurcation. Instead, a bifunctional acetaldehyde/alcohol dehydrogenase, AdhE, is used that catalyzes two consecutive oxidation steps (75). First, AdhE oxidizes ethanol to acetaldehyde. This intermediate is immediately oxidized by the same enzyme to acetyl-CoA. Both oxidation steps utilize NAD^+ as electron acceptor. Oxidation of acetaldehyde to acetyl-CoA with NAD^+ is a highly exergonic reaction ($\Delta G^{0\prime}$ of -17 kJ/mol) (54, 55). In equilibrium, the concentration of acetaldehyde is very low, which makes the first oxidation of ethanol to acetaldehyde thermodynamically feasible (Fig. 4A, inset). Catalyzing both the endergonic and the exergonic reactions by the same enzyme might be advantageous as it would keep the acetaldehyde level low by directly oxidizing the intermediate without releasing it out of the enzyme. In sum, ethanol oxidation to acetyl-CoA is only a slightly endergonic reaction (reaction 14; $\Delta G^{0\prime}$ of $+5$ kJ/mol).
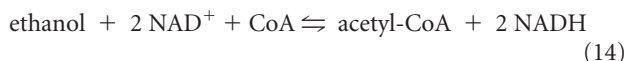
$$\text{ethanol} + 2\,NAD^+ + \text{CoA} \rightleftharpoons \text{acetyl-CoA} + 2\,NADH \quad (14)$$
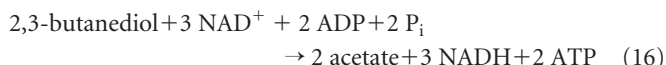
Acetyl-CoA is converted to acetyl-phosphate by a phosphotransacetylase, followed by a transfer of the phosphate group to ADP, resulting in formation of acetate and generation of 1 ATP by SLP. Reoxidation of both NADH molecules is catalyzed by the WLP. In *A. woodii*, the WLP utilizes 2 NADH, 1 reduced ferredoxin, and 1 H_2 (corresponding to 0.5 NADH and 0.5 reduced ferredoxin when generated by the electron-bifurcating hydrogenase) to reduce 2 molecules of CO_2 to acetate (Fig. 1). To balance the oxidative and reductive modules, the Rnf complex has to catalyze the unfavorable electron transfer from NADH to ferredoxin. Ethanol oxidation is, therefore, the second energy-conserving metabolic pathway in acetogens that involves reverse electron transfer driven by a chemiosmotic ion (Na^+) gradient. To establish this gradient, part of the ATP produced by the acetate kinase reaction has to be reinvested to drive the membrane-bound ATP synthase. In sum, *A. woodii* converts ethanol to acetate according to equation 15.

$$2\,\text{ethanol} + 2\,CO_2 + 1.5\,\text{ADP} + 1.5\,P_i \rightarrow$$
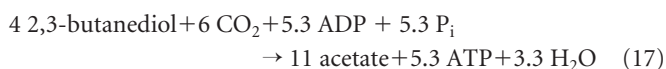$$3\,\text{acetate} + 1.5\,\text{ATP} + 1.5\,H_2O \quad (15)$$
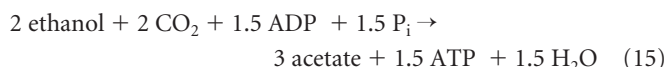
The thermodynamic efficiency of 82% for this pathway is the highest for all conversions we have addressed and far above the typical value of 20 to 50% for anaerobic metabolisms (3). The downside of this high efficiency is the relative high substrate concentration that is necessary for growth, described as a high threshold concentration (the minimal substrate concentration that allows growth and is a result of the released amount of energy, which decreases if the substrate concentration is lowered). It has also experimentally been described that *A. woodii* requires very high HCO_3^- concentrations of a minimum of 60 mM (optimum, 120 mM) to grow with ethanol as the substrate (76).

A. woodii is not restricted to monovalent alcohols such as ethanol; it can also grow via the oxidation of divalent alcohols, such as 2,3-butanediol or 1,2-propanediol. Both pathways have recently been elucidated with the model acetogen *A. woodii* (46, 77). 2,3-Butanediol is the natural product of butanediol fermentation of carbohydrates, a process typical for some enterobacteria, such as *Enterobacter* or *Klebsiella* species (78). Some acetogens, such as *A. woodii*, can conserve energy for growth by the further oxidation of 2,3-butanediol to acetate. CO_2 can be used as the electron acceptor, as it is reduced to an additional acetate via the WLP. The pathway of 2,3-butanediol oxidation in *A. woodii* is shown in Fig. 4B (77). In the oxidative part, 2,3-butanediol is oxidized to acetoin by an NAD^+-dependent 2,3-butanediol dehydrogenase. In the next step, a multimeric acetoin dehydrogenase oxidizes and cleaves acetoin into acetyl-CoA and acetaldehyde. The architecture of the acetoin dehydrogenase of *A. woodii* resembles bacterial pyruvate dehydrogenase or α-ketoglutarate dehydrogenase enzyme complexes (79, 80). In the next step of the pathway, acetaldehyde is oxidized to acetyl-CoA by an NAD^+-dependent alcohol dehydrogenase. Finally, both acetyl-CoA are converted to acetate, which is connected to the synthesis of ATP by SLP (Fig. 4B) (77). The oxidative module can be summarized as shown in reaction 16.

$$2,3\text{-butanediol} + 3\,\text{NAD}^+ + 2\,\text{ADP} + 2\,\text{P}_i$$
$$\rightarrow 2\,\text{acetate} + 3\,\text{NADH} + 2\,\text{ATP} \quad (16)$$

In the second part of the metabolic scheme, the WLP reduces two CO_2 to acetate to reoxidise NADH and equalize the redox balance. This is again only possible by reverse electron transfer from NADH to reduced ferredoxin catalyzed by the Rnf complex and energized by the chemiosmotic sodium ion gradient (77). Therefore, the 2,3-butanediol pathway is the third example of a metabolic route in acetogens, where the Rnf complex utilizes energy to drive an unfavorable reaction. To build up the ion gradient, part of the ATP produced by SLP must be reinvested. As shown in Fig. 4B, complete conversion of 2,3-butanediol by *A. woodii* is catalyzed with the stoichiometry shown in reaction 17.

$$4\,2,3\text{-butanediol} + 6\,CO_2 + 5.3\,\text{ADP} + 5.3\,\text{P}_i$$
$$\rightarrow 11\,\text{acetate} + 5.3\,\text{ATP} + 3.3\,H_2O \quad (17)$$

The energy released by this reaction (calculated without phosphorylation of ADP) is -89 kJ/mol 2,3-butanediol (54, 55). Therefore, the thermodynamic efficiency is 66% (1.33 ATP/2,3-butanediol multiplied by -43.9 kJ/mol ATP, divided by -89 kJ/mol 2,3-butanediol), a value again only possible with flavin-based electron bifurcation. After reflecting on our current knowledge of the heterotrophic metabolism of acetogens, we want to address the ecological consequences and biotechnological potential of these pathways.

ECOLOGICAL SIGNIFICANCE OF DIVERSE HETEROTROPHIC METABOLISM

What is the place of acetogens in an anaerobic ecosystem? The crux of the question touches the competiveness of acetogens. Members of this group of organisms have been isolated from numerous, and very different, anaerobic ecosystems: gastrointestinal tracts of mammalians and termites, sediments, soils, acidic habitats, and ecosystems with high salt concentrations or high temperatures are just a few examples (21, 81–87). There is also more and more evidence accumulating that acetogens represent quantitatively important components in the respective ecosystems (88–92). However, from simple thermodynamic considerations, the physiological role of acetogens is less than clear. Figure 5 illustrates the typical anaerobic food web in a sulfate-poor environment, such as a freshwater sediment. In this trophic chain, there is only one conversion that is exclusively occupied by acetogens: interconversion of H_2 plus CO_2 and acetate. However, from the simple scheme, this step seems to be not essential for complete mineralization of carbon. In addition, the same substrates are utilized by methanogenic archaea. The larger amount of energy from the methanogenic reaction ($4\,H_2 + CO_2 \rightarrow CH_4 + 2\,H_2O$; $\Delta G^{0\prime}$ of -131 kJ/mol) allows growth and energy conservation of methanogens at much lower substrate concentrations than necessary for acetogenesis, thus outcompeting them (93). The competition is tightened when sulfate is present (not shown in the figure). H_2 is a common substrate for sulfate reducers that can outcompete both acetogens and methanogens in the presence of sulfate, due to the even larger amount of available energy (94). Therefore, if sulfate is present, sulfate reducers should be the dominant H_2-consuming species, and if not, methanogens should take over. So, where do acetogens come into play? The currently predominant assumption is that acetogens are competitive due to their tremendous substrate range (95). If considering a single substrate, in
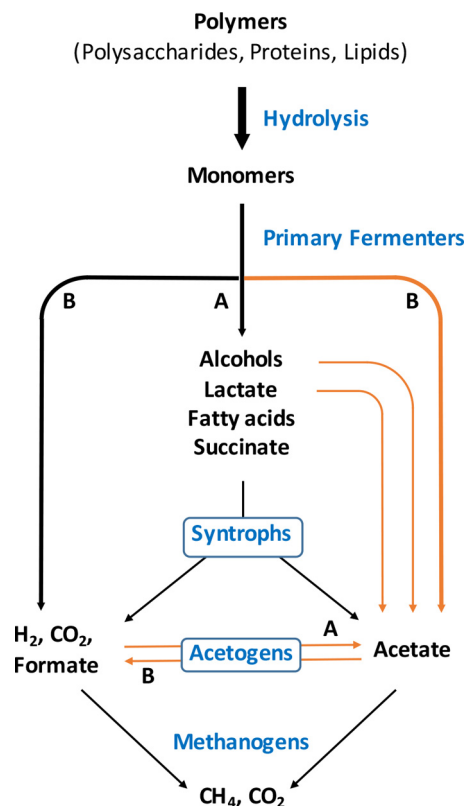

FIG 5 Role of acetogens in the anaerobic food web. The methanogenic degradation in sulfate-poor environments, such as freshwater sediments, is characterized by initial hydrolysis of polymers followed by the conversion of monomers by primary fermenting bacteria. Alcohols, short-chain fatty acids, and other intermediary products are converted by secondary fermenting bacteria (syntrophs) to acetate, H_2, and CO_2 before these substrates are used by methanogenic archaea for methanogenesis. Under low hydrogen pressure in a well-balanced fermentation, the major routes are labeled with a B. Higher hydrogen pressures lead to an increased flux through the routes marked with an A. Conversions where acetogens can participate are shown in orange.

most cases one can find a more specialized organism that should outcompete an acetogen, based on thermodynamic grounds. However, in complex and, conceivably, rapidly changing environments, the remarkably flexible metabolism of acetogens offers many advantages (95). The illustrated anaerobic food web of Fig. 5, highlighting all positions where acetogens can participate, shows that they might play a much more important role. They can take over functions of primary fermenters when utilizing carbohydrates or specialized substrates such as methoxylated compounds. They also can convert substrates such as alcohols or lactate that are otherwise restricted to syntrophs (72). All this metabolic potential separates acetogens from methanogens that are restricted to H_2 plus CO_2, carbon monoxide, formate, methanol, methylamines, and acetate and, to a lesser extent, from sulfate reducers that can, viewing all members of this group together, use many different substrates as well (93, 94). However, most single sulfate-reducing species have a limited substrate spectrum and often only use H_2, short-chain fatty acids, and ethanol (96).

The ability to utilize many different substrates and the relative high ATP yields and thermodynamic efficiencies might explain the natural abundance of acetogens. Different advantages might arise from this ability to utilize different substrates: acetogens can

use substrates that are not used by sulfate reducers or methanogens and thus are "leftover" substrates. Otherwise, they can also pool the energy of different substrates that are used simultaneously (95). In this scenario, they would need lower turnover rates and also a lower supply of a single substrate compared to specialized organisms utilizing only one substrate alone. Acetogens can also cooperate with methanogens, as exemplified by the complete conversion of carbohydrates by a coculture of *A. woodii* and *Methanosarcina barkeri*, in which the methanogen removes acetate in favor of the acetogen (97), or the interspecies H_2 transfer described above between *A. woodii* and an H_2-consuming methanogen that results in release of H_2 from the acetogen instead of acetogenesis via the WLP. Another fascinating feature that we have completely neglected so far is reverse acetogenesis. As indicated in Fig. 5 by the arrows in both directions between H_2 plus CO_2 and acetate, whereas acetogens produce acetate under high H_2 pressures, some acetogens can reverse this process if the H_2 pressure becomes very low (98, 99). In this case, acetate is oxidized, presumably by reversing the WLP (26). This adds to the remarkable flexibility that extends beyond a huge number of potential substrates to the possibility of completely reversing a metabolic pathway, depending on the environmental conditions. Altogether, acetogens seem at first glance very uncompetitive against very specialized organisms, such as methanogens, and it is unlikely that the *in situ* function of acetogens is predominantly autotrophic growth with H_2 plus CO_2, a feature that is mainly connected with acetogens. Exemptions are some special environmental conditions, such as low pH or low temperatures, where methanogens are inhibited (72). The competeiveness more likely results from the metabolic flexibility and the energetic optimization of the metabolism toward heterotrophic substrates. The recent experimental proceedings toward a detailed understanding of the conversion of the multifaceted substrate spectrum of acetogens give a metabolic explanation for this diversity.

ACETOGENESIS AS A MODULE FOR USE IN SYNTHETIC BIOLOGY

The unique metabolism of acetogens can also be utilized for biotechnological purposes. For example, in the process called anaerobic nonphotosynthetic (ANP) mixotrophic fermentation, the ability of acetogens to utilize CO_2 released during carbohydrate fermentation is used to improve yield coefficients (100, 101). Carbohydrate fermentations, e.g., from glucose to acetate or ethanol, involve decarboxylation reactions that result in the loss of carbon into CO_2. This has a large impact on the maximum mass yield that can be obtained. From 1 mol of glucose, a maximum of 2 mol of acetate or ethanol can be produced. The remaining carbon is lost in 2 mol of CO_2 that results from oxidative decarboxylation of pyruvate. This loss has been thus far mostly disregarded; however, today's increased requirement to minimize CO_2 emissions as well monetary pressures for biofuel production to successfully compete with fossil fuel demand that this inefficiency be taken into account. For successful commercialization and competitive biofuel production, all by-products must be utilized to achieve competitive product prices. Since the discovery of the acetogen *M. thermoacetica* 70 years ago, it is known that acetogens can ferment glucose without CO_2 emissions and convert it completely to 3 acetates, thus increasing the theoretical product yield by 50% (6). This is not achieved by replacing classical glycolysis with a different pathway for carbohydrate oxidation, but by recapturing the

evolved CO_2 by fixing it via the WLP (Fig. 3A). CO_2 can thus be regarded as the second substrate and the process as mixotrophic fermentation. Looking beyond carbohydrate fermentation, the WLP could theoretically be coupled to every CO_2 releasing pathway to recapture the by-product. Fixation of CO_2 can be catalyzed by, to date, six different known pathways (10). However, most of these pathways need energy (ATP). A given fermentation process must have a positive ATP yield to provide energy for growth and cell maintenance. Since fermentations are connected with energy limitation and thus very low ATP yields compared to respiratory processes, coupling of these pathways to ATP-consuming CO_2 fixation pathways is problematic. Of the six known natural pathways of CO_2 fixation, the WLP requires the least energy (10). The net ATP gain of this pathway by SLP is zero when acetyl-CoA is converted to acetate, as discussed above; however, when converting acetyl-CoA into biomass, this pathway consumes less than 0.5 ATP per fixed CO_2 molecule. For biotechnological purposes, we are not interested in the fixation of CO_2 into biomass, but instead into product formation. If acetate is produced, the ATP/CO_2 ratio is 0, and thus this pathway can be combined with every fermentation without affecting the ATP output. This makes the WLP ideally suited for capturing CO_2 to increase product yields. On the downside, ANP mixotrophic fermentation is limited by the redox balance. Reduction of 2 CO_2 to acetate requires 8 electrons. Therefore, the first requirement for a pathway coupled to the WLP is the release of 8 electrons per 2 CO_2 molecules, to allow complete CO_2 fixation. This is the case for oxidation of glucose to 2 acetate molecules via typical fermentations. If looking for other end products, ANP mixotrophy is limited to end products with the same oxidation state as acetate. Otherwise, glucose oxidation does not provide enough electrons. Therefore, ANP mixotrophy cannot increase the mass yield for the production of biofuels such as ethanol or butanol.

Can we circumvent the electron limitation? With use of carbohydrates as the sole substrate, this is not possible. However, supplying additional electron donors such as H_2 or electrons provided by a cathode for direct electron uptake overrides this limitation. In this case, H_2 provides an unlimited source of reducing power. As pointed out earlier, acetogens naturally harbor hydrogenases; thus, the enzyme machinery for this approach is already available. Glucose fermentation coupled to the WLP in the presence of hydrogen has a theoretical carbon efficiency of 100% (ignoring biomass formation) according to reaction 18 ($\Delta G^{0'}$ of -337 kJ/mol [54, 55]).

$$\text{glucose} + 6\,H_2 \rightarrow 3\,\text{ethanol} + 3\,H_2O \qquad (18)$$

With the metabolism of *A. woodii* as the basis, this fermentation provides 3.1 ATP per glucose molecule (Fig. 6). Glucose fermentation with an H_2 supply also increases the product yield for production of other reduced products, such as isopropanol, butanol, or pentanol. This technology can theoretically be used for the production of any product from glucose, as long as the ATP yield has a positive value. This value can be calculated from the 2 ATPs produced by glycolysis plus the ATP produced by the Rnf complex from NADH reduction with reduced ferredoxin, subtracted from the ATP consumed for product synthesis. Our detailed understanding of the WLP in model organisms such as *A. woodii* is the basis for synthetic biology approaches to establish ANP mixotrophic fermentation. Energy limitation and the requirement for a detailed understanding of the electron flow necessitate a detailed
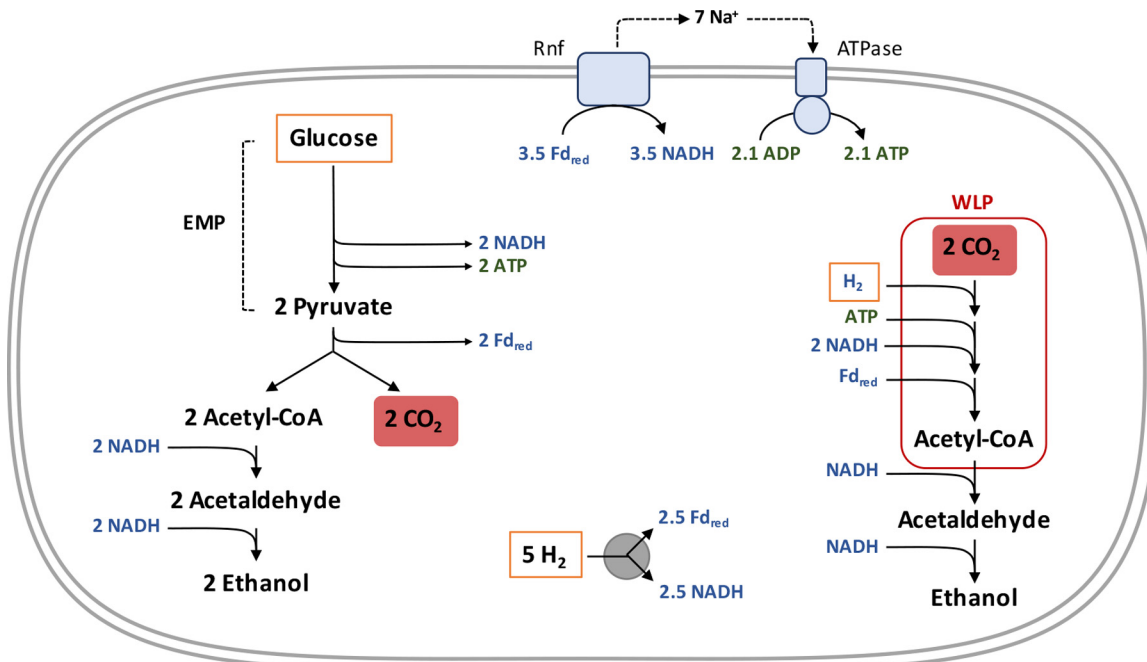
FIG 6 Synthetic biology approach to increase the yield coefficient of ethanol formation. Coupling the WLP to the formation of ethanol from glucose can increase the theoretical maximum yield from 2 to 3 molecules of ethanol formed per glucose molecule. The necessary energy is provided by SLP and chemiosmotic energy conservation via the Rnf complex. Electrons can be provided, for example, by molecular hydrogen or by direct electron uptake from a cathode. EMP, Embden-Meyerhof-Parnas pathway (glycolysis); Fd_{red}, reduced ferredoxin (reduced by 2 electrons).

understanding of every single reaction to calculate the viability of an intended production pathway.

We can also turn the synthetic biology approach the other way around. Can we introduce the WLP into production hosts? So far it has, to our knowledge, never been shown that the complete WLP can be produced heterologously. This would be a fantastic experiment to define the minimal set of genes necessary for the WLP. Although most models of the WLP indicate a thorough understanding of the pathway, the reality lags behind. If we just look in the gene cluster coding for all enzymes of the WLP, for example in the best understood acetogen, *A. woodii*, there are genes scattered between the genes coding for known WLP enzymes that have no assigned function. Do these genes code for essential functions that have not been identified yet? Assembling a synthetic acetogen by introducing the minimal set of genes into a nonacetogenic bacterium would push our understanding far ahead.

CONCLUDING REMARKS

In summary, the foundation of the metabolic flexibility of acetogens might be represented by a very modular metabolism, which allows the utilization of many different substrates with a minimal set of enzymes. In most cases, the substrate is oxidized to acetyl-CoA. This central intermediate is then converted to acetate as the end product, coupled to SLP and thus providing ATP. Often unappreciated in metabolic considerations is the requirement for a balanced redox stoichiometry. In some cases this can be neglected, as in the case of aerobic organisms, for which most electrons are transferred by NADH and oxygen is available as an unlimited oxidant. In the metabolism of anaerobes, redox balancing is very important and not as easy to achieve, since suitable electron ac-

ceptors are limited and the low redox potentials of the electron acceptors often lead to thermodynamic barriers that permit oxidation of some substrates/intermediates. Acetogens evolved an extremely elegant way to solve the problem of redox balancing which is the basis to the exploitation of many substrates. This is done by soluble, electron-bifurcating enzymes or membrane-bound processes. The WLP is used to reoxidize the electron carriers that are reduced during substrate oxidation. The detailed analysis of all enzymes of this pathway revealed that it is not coupled to the membrane potential and is, from the perspective of ATP synthesis, energy neutral. If combining the electron-bifurcating hydrogenase and the WLP, the only input in this pathway is NADH and reduced ferredoxin. Another very important feature of acetogens is that energetic barriers, such as H_2 production from NADH, are solved by flavin-based electron bifurcation and not by coupling to ATP hydrolysis or coupling to the membrane potential. This preserves the isolation of this pathway from other processes and keeps it as a sole means for redox balancing. The next hurdle is to balance the ratio of NADH and reduced ferredoxin, which are generated in different amounts from different substrates. The Rnf complex is introduced as an energy-coupled "redox equalizer" or "switch" (Fig. 2B). It always adjusts the right ratio of reduced ferredoxin and NADH, independent of the substrate oxidized. If this adjustment allows for energy conservation, the Rnf complex serves as a coupling site for additional ATP synthesis. If the ratio is unfavorable, the Rnf complex serves as an engine to drive the electron flow "uphill." These two modules, WLP for reoxidation and Rnf complex/electron-bifurcating hydrogenase as the switch, can now be combined into dozens of different oxidation modules.

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